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ANTIOXIDANT CAPACITY AND METABOLIC RESPONSE OF RED STRIPED SNAPPER Lutjanus erythropterus FED DIET WITH ASTAXANTHIN AND/OR OXIDIZED OIL

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ANTIOXIDANT CAPACITY AND METABOLIC RESPONSE OF RED STRIPED SNAPPER *Lutjanus erythropterus* FED DIET WITH ASTAXANTHIN AND/OR OXIDIZED OIL

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Key words: antioxidant capacity, astaxanthin, metabolic response, oxidized oil.

ABSTRACT

This study evaluated the effects of astaxanthin (AX) on antioxidant capacity, metabolic response, and liver AX concentration of red striped snapper (*Lutjanus erythropterus*) (20.05 ± 1.65 g) fed with a diet with or without high dosage of oxidized oil (OX). The commercial diet served as control (C). Fish were fed with a diet containing 240 mg/kg AX, 100 ml/kg OX, or combination of 240 mg/kg AX and 100 ml/kg OX (AX + OX) for a day. Antioxidant capacity (superoxide dismutase, glutathione peroxidase, and glutathione reductase) and metabolic response (glucose, triglycerides, and lactate) were monitored at 0, 6, 12, 24, 48 and 72 h while liver AX concentration was analyzed at 72 h after feeding. AX- and AX + OX-fish had more improved antioxidant capacity and metabolic response than C- and OX-fish at 6 and 12 h. Moreover, OX-fish had higher glutathione reductase, glucose, triglycerides, and lactate concentrations than AX-fish at 6 and 12 h. Treatments that received AX showed higher liver AX concentration as expected. Overall, this study demonstrated that supplementation of AX in the diet significantly stabilizes both antioxidant capacity and metabolic responses of L. erythropterus under a prooxidant circumstance associated with oxidized fish oil intake.

I. INTRODUCTION

Animals have a natural response to counteract in vivo oxidation to prevent tissue oxidation and maintain homeostasis (Finkel and Holbrook, 2000). Consequently, the health of aquatic organisms is linked to overproduction of reactive oxygen species (ROS) such as superoxide anion radical ($\bullet O_2$-), hydroxyl radical ($\bullet$OH), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) (Secombes and Fletcher, 1992; Lygren et al., 1999). Most cells also acquire the relevant protective mechanisms to maintain the lowest possible levels of ROS inside the cell (Finkel and Holbrook, 2000; Hermes-Lima and Zenteno-Savín, 2002). Various antioxidant compounds, such as vitamin A, C, E, astaxanthin, and antioxidant defense enzymes, are present in fish system. These protect cell membranes against the production of free radicals (Shimidzu et al., 1996).

Free radical scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase, and glutathione reductase (GR) are the first line of defense of fishes against oxidative injury. SOD, a cytosolic enzyme that is specific for scavenging radicals, is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. It catalyzes reactive oxygen molecules to H_2O_2 that is consequently converted to water by catalase (Hartog et al., 2003). The GPx family is part of the body's antioxidant system which reduces organic hydroperoxides while oxidizing glutathione (GSH) to glutathione disulfide (GSSG) (Brigelius-Flohe, 1999). GPx also scavenges lipid hydroperoxides and associated enzymes such as GR which acts to maintain the levels of reduced glutathione (Mourente et al., 2002).

Physiological response enables fish to respond appropriately to stressors and maintain homeostasis (Carr and Norris, 2006). Oxidized oil can impair the physiological response (Mourente et al., 2002) by damaging components within the signaling pathway through mechanisms such as physical stress (Dorval and Hontela, 2003). Previous study indicated that glucose, triglycerides, and lactate are commonly used as functional parameters for evaluating plasma metabolites of fish (Barton, 2002). Increased metabolism by physical stress can be associated with higher ROS generation that affects oxidative status.

Carotenoids are potent antioxidants that help to inactivate free radicals produced from normal cellular activity and biological and environmental stress (Chew, 1995). AX possesses

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good singlet oxygen quenching properties and may serve as an antioxidant in systems containing unsaturated fatty acids (Martin, 1999). It also showed strong activity as an inhibitor of lipid peroxidation mediated by active forms of oxygen and was considered to be a "super vitamin E" (Miki, 1991). It was reported that enhancement of resistance to hypoxia stress (Chien et al., 1999; Pan et al., 2010a), salinity and thermal stress (Chien et al., 2003), ammonia stress (Pan et al., 2003a; Pan et al., 2010b) and pathological stress (Pan et al., 2003b) was associated with an increase in dietary and body AX content. The good antioxidant properties of AX may relieve oxidative stress, provide beneficial biological effects (Angeles et al., 2009; Zhang et al., 2013), and have also been linked to health as reviewed by Higuera-Ciapara et al. (2006) and Hussein et al. (2006).

Marine fish diets contain large amount of highly unsaturated fatty acids (HUFA) which are easily oxidized. The oxidized HUFA might lead to oxidative stress in fish (Nakano et al., 1999). It was reported that feeding of OX has negative effects on fish histopathology and hematology (Huang and Huang, 2004). High oil oxidization also led to the weak antioxidant capacity (low serum SOD and GPX) and poor survival against *Aeromonas hydrophila* infection (Wang et al., 2015). Moreover, previous studies have showed that rancid oil in fish diet can cause growth suppression (Baker and Davies, 1996), lipid peroxidation (Tocher et al., 2003), skeletal deformity (Lewis-McCrea and Lall, 2007), and animal health deterioration (Hamre et al., 2001). Aside from its pigment-enhancing property, AX is commonly supplemented to fish diet to act as a powerful antioxidant against oxidative stress (Niu et al., 2011).

In pharmacokinetics studies, responses to nutraceuticals can be observed after injection or short-period feeding of high dosage. The maximum level of AX in the serum can be reached within 24 h after one-day feeding (Gobantes et al., 1997; Choubert et al., 2005). Serum AX concentrations in rainbow trout (*Oncorhynchus mykiss,* Walbaum) reached the peak at 18 h after feeding and its mean retention time recorded at 56.4 h (Gobantes et al., 1997). Therefore, this study was carried out to investigate if AX could improve antioxidant capacity and stabilize metabolic response of red striped snapper *Lutjanus erythropterus* (Bloch) fed diet with or without OX after feeding for one day. In this sense, plasma SOD, GPx, and GR were studied as well as glucose, triglycerides, and lactate in plasma. Liver AX concentration of *L. erythropterus* was last determined at 72 h after feeding the treatment.

II. MATERIALS AND METHODS

1. Fish, Diet, and Experimental Condition

Red striped snapper (20.05 ± 1.65 g) were obtained from a commercial farm in Pingtung, Taiwan. They were acclimated indoors for two weeks in a circular tank (2 m D × 0.5 m H) and fed with a commercial diet that is 5% body weight (BW). Fish were fed at 08:00, 15:00, and 22:00 h daily. After acclimation, the fish were individually weighed and transferred into 20-L glass aquaria. These were then fasted and acclimated in an aquarium condition for 3 days prior to the administration of a single dose *ad libitum* feeding trial. The starvation procedure was carried out prior to blood sampling to stabilize the plasma metabolites with respect to short term fluctuations of nutrients after feeding the fish (Lygren and Waagbø, 1999).

This study had four treatments. Each treatment had six fish per sampling time. The basal (commercial) diet served as the control. Fish were fed with a diet containing 240 mg/kg AX, 100 ml/kg OX, or combination of AX + OX (240 mg/kg AX and 100 ml/kg OX) for one day. The OX was prepared by the introduction of vigorous bubbling air through fish oil at 40°C for 120 h to raise its peroxide value (Nakano et al., 1999) or intermediates in the autoxidation reaction. Autoxidation is a free radical reaction involving oxygen that leads to deterioration of fats and oils which form off-flavors and off-odors. AX (10% Carophyll Pink CWS (DSM Nutritional Products, Basel, Switzerland)) was dissolved in distilled water and put in an ultrasonic cleaner for 10 minutes. Agar solution was prepared by adding 6 g of agar to 100 ml distilled water, heated to dissolve the agar, and then cooled down to below 40°C. The AX solution was then mixed in agar solution and sprayed to 1000 g control diet and dried in a forced-air oven at 40°C for 24 h. All diets were stored in dark plastic containers at -20°C to avoid deterioration prior to use.

Fresh fish oil (100 ml/kg) and agar solution were added to the non-OX and non-AX diet, respectively, to counterbalance the contents of different treatments. The basal diet is a commercial marine fish diet composed of 44% crude protein, 3% lipid, 16.5% ash, 1.2% fiber, and 11% moisture.

Each aquarium was provided with aerator and natural photoperiod. Bottom debris was siphoned out and one-third of the water was replaced daily. Water quality parameters such as dissolved oxygen (5.18-6.32 mg/l), temperature (27.2-28.3°C), and pH (7.1-7.6) were monitored and kept within safe levels.

2. Antioxidant Capacity and Metabolic Response

Six fish from each treatment per sampling time were sampled. Fish were anaesthetized with tricane methane sulfonate (MS-222; 100 mg/l) and heparinized blood was withdrawn from the caudal vessel using a 1-ml sterile syringe with 25 gauge needle 6 h after feeding. The same procedure was followed using different set of fish per treatment per sampling time at 12, 24, 48 and 72 h after feeding. Six fish with no treatment were used as the initial control. Blood was centrifuged for 5 min at 1800 g and the plasma was drawn off and immediately frozen (-20°C) for later analysis for antioxidant capacity and metabolic response.

Antioxidant capacity was analyzed spectrophotometrically using a U-2000 spectrophotometer at 37°C at 505 nm absorbance for SOD and 340 nm absorbance for GPx and GR. The volumes of plasma used in these assays were 25 µl for SOD, 40 µl for GPx, and 200 µl for GR. All assays were performed within 5 h after sampling using Randox Laboratories kits

Table 1. Average activities of plasma antioxidant capacity of red striped snapper *Lutjanus erythropterus* at 6, 12, 24, 48, and 72 h after being fed with AX- and OX-supplemented diet.

Time (h)	Parameters (U/ml)	Astaxanthin[1] (AX)		Oxidized oil[2] (OX)		AX x OX Interaction[3] (Pr > F)	M.S.E.[4]
		No	Yes	No	Yes		
6	SOD[5]	0.468^{a} (0.022)	0.356^{b} (0.036)	0.384^{x} (0.023)	0.494^{x} (0.040)	0.32	1×10^{-2}
	GPx[6]	0.206^{a} (0.007)	0.191^{b} (0.004)	0.189^{y} (0.005)	0.208^{x} (0.006)	0.26	4×10^{-4}
	GR[7]	0.014^{a} (0.001)	0.012^{b} (0.001)	0.013^{x} (0.001)	0.014^{x} (0.001)	0.74	9×10^{-6}
12	SOD	0.601^{a} (0.062)	0.329^{b} (0.028)	0.406^{x} (0.038)	0.524^{x} (0.081)	0.27	2×10^{-2}
	GPx	0.310^{a} (0.011)	0.271^{b} (0.005)	0.275^{y} (0.007)	0.0306^{x} (0.011)	0.18	3×10^{-4}
	GR	0.016^{a} (0.001)	0.014^{b} (0.001)	0.014^{y} (0.001)	0.017^{x} (0.001)	0.81	6×10^{-8}
24	SOD	0.419^{a} (0.019)	0.410^{a} (0.014)	0.408^{x} (0.018)	0.422^{x} (0.015)	0.98	3×10^{-6}
	GPx	0.023^{a} (0.007)	0.200^{a} (0.005)	0.196^{x} (0.005)	0.207^{x} (0.006)	0.63	8×10^{-5}
	GR	0.012^{a} (0.0003)	0.011^{a} (0.0004)	0.011^{x} (0.0004)	0.012^{x} (0.0003)	0.35	1×10^{-6}
48	SOD	0.395 (0.019)	0.363^{a} (0.016)	0.366^{x} (0.017)	0.393^{x} (0.019)	0.53	1×10^{-3}
	GPx	0.206^{a} (0.005)	0.202^{a} (0.005)	0.196^{y} (0.005)	0.212^{x} (0.00074)	0.88	5×10^{-6}
	GR	0.011^{a} (0.0003)	0.011^{a} (0.0004)	0.011^{x} (0.0004)	0.012^{x} (0.0004)	0.66	2×10^{-7}
72	SOD	0.367^{a} (0.021)	0.381^{a} (0.018)	0.354^{x} (0.018)	0.394^{x} (0.022)	0.91	8×10^{-5}
	GPx	0.204^{a} (0.005)	0.204^{a} (0.004)	0.198^{y} (0.004)	0.211^{x} (0.003)	0.77	1×10^{-5}
	GR	0.011^{a} (0.0005)	0.011^{a} (0.0004)	0.011^{x} (0.001)	0.011^{x} (0.0004)	0.73	2×10^{-7}

Under the same main factor, means (S.E.) without a common superscript are significantly different ($p \leq 0.05$).

[1] Diets were supplemented with astaxanthin and with and without oxidized oil.

[2] Diets were supplemented with oxidized oil and with and without astaxanthin.

[3] Interaction between astaxanthin and oxidized oil.

[4] Mean standard error.

[5] Superoxide dismutase.

[6] Glutathione peroxidase.

[7] Glutathione reductase.

according to manufacturer's instructions (Biagini et al., 1995). One unit of activity was expressed in U/ml.

Commercial kits were used to determine glucose (Randox, GOD-PAP), lactate (Randox, PAP), and triglycerides (Randox, GPO-PAP). Methods were adapted to a microplate, using 20 μL plasma and 200 μl enzyme chromogen reagent (Palacios et al., 2000). Glucose, triglycerides, and lactate concentrations were expressed in mg/dl plasma.

3. Liver AX Concentration Analysis

Six fish from each treatment were sampled for analysis of liver AX concentration at 72 h after feeding. The dissected liver was freeze-dried using a refrigerated air dryer (model FD-12-6P-D, Kingmech, Tuncheng, Taiwan R.O.C.) and ground using a porcelain mortar and pestle. Samples were then placed into a polypropylene centrifuge tube and 20 ml acetone with 0.05% butylated hydroxytoluene (BHT) was added as an antioxidant and solvent. The mixture was homogenized using Polytron PT MR-3000 homogenizer (Kinematica AG, Littau, Switzerland) at 8000 *g* for 1 min. The samples were then centrifuged at 4°C, 10000 g for 15 min. The resulting liquid phase was transferred into a 250 ml separatory funnel, partitioned with 30 ml n-hexane, and washed twice with 25 ml 10% NaCl solution to remove residual acetone. The extract was put into a rotary evaporator (Rotavapor Model R114, Büchi, Switzerland) at 30°C in a water bath (Model B480, Büchi, Switzerland) to reduce the volume. The samples were further dried from excess water using nitrogen gas (N_2) and filtered through a 0.22 μm Millipore filter. Samples of 3-ml were stored in brown vials and finally allocated inside an autosampler (Model L-7200, Hitachi, Japan) prior to high performance liquid chromatography (HPLC) analysis.

HPLC analysis was done using a Hitachi L-6200 pump, silica column 250 × 4.6 mm (LUNA 5 μ SILICA, Phenomenex Inc.), Hitachi L-4250 UV/Vis detector, and Hitachi D-2000 Chromato-integrator. This system was controlled by a chromatographic data system (Scientific Information Services Corporation). The program of solvents at mobile phase include: mixture A composed of 490 ml n-hexane, 5 ml dichloromethane, and 5 ml isopropyl alcohol; mixture B com-

posed of 460 ml n-hexane, 5 ml dichloromethane, and 35 ml isopropyl alcohol. The absorption maximum was set to 470 nm and the work volume at 1 ml/min.

Total AX (TA) was determined as the sum of monoester AX (MA), diester AX (DA), and free AX (FA).

4. Statistical Analysis

The data on antioxidant capacity, metabolic response, and liver AX concentrations were subjected to Kolmogrov- Smirnov for normality and Cochran's test for homogeneity of variance. The data were homogenous and showed normal distribution. Two-way analysis of variance (ANOVA) was then performed to find out the main effects of AX and OX and their interaction effects on antioxidant capacity (SOD, GPx, and GR) and metabolic response (glucose, triglycerides, and lactate) at 0, 6, 12, 24, 48, and 72 h after feeding. One-way ANOVA was also performed to determine the effects of AX and/or OX on antioxidant capacity and metabolic response at each sampling time and on liver AX concentration at 72 h. Duncan's Multiple Range Test was used to compare differences among treatments. Correlation was used to analyze relationships among antioxidant capacity, metabolic response, and liver AX concentration. The level of significance set for all analyses was $p \leq 0.05$.

III. RESULTS

1. Antioxidant Capacity

Main effects of AX and OX and their interaction on antioxidant capacity are presented in Table 1. AX-fed fish (AX and AX + OX had significantly lower SOD, GPx, and GR than the non-AX-fed fish (C and OX) at 6 and 12 h after feeding. There were no differences found in all antioxidant capacity since 24, 48, and 72 h. On the other hand, OX-fed fish (OX and AX + OX) resulted in significantly higher GPx compared to non-OX-fed fish (C and AX) at 24 h. Moreover, OX-fed fish had significantly higher GR than non-AX-fed fish (AX and AX + OX) at 6 and 12 h. However, AX and OX had no observed significant interaction effect on antioxidant capacity.

Four treatments had significant effects on SOD, GPx, and GR only at 6 and 12 h (Fig. 1). AX- and AX + OX-fish showed lower SOD, GPx, and GR compared to those OX- fish at 6 and 12 h. The difference between AX and OX even increased at 12 h. However, no difference in SOD, GPx, and GR was found from 24 h onwards.

2. Metabolic Response

The main effects of AX and OX and their interactions on plasma metabolic response are presented in Table 2. AX-fed fish (AX and AX + OX) had significantly lower glucose concentration than the non-AX-fed fish (C and OX) at 6 and 12 h but had lower triglycerides and lactate concentrations at 12 and 6 h, respectively. There were no differences found in all metabolic response starting 24 h to 48 and 72 h. On the other hand, OX-fed fish (OX and AX + OX) had significantly higher glucose and triglyceride concentrations at 12 h and lactate

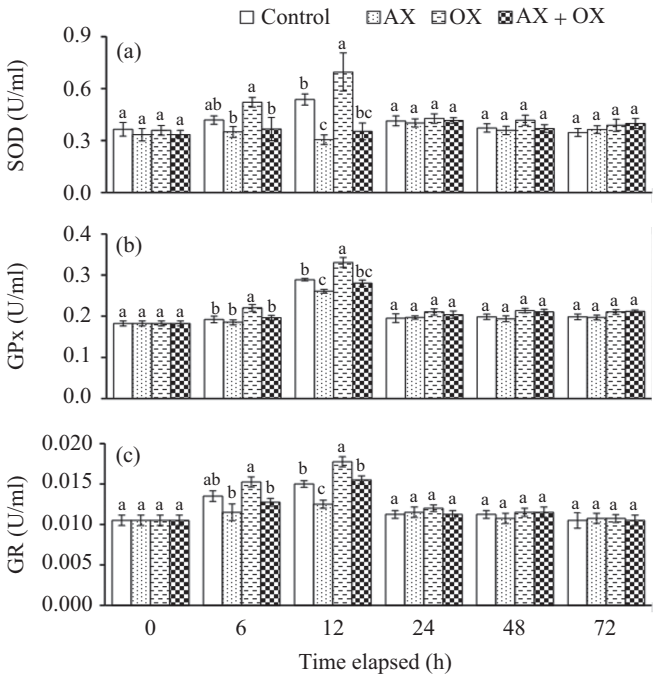

Fig. 1. Mean (S.E.) superoxide dismutase (SOD) (a), glutathione peroxidase (GPx) (b), and glutathione reductase (GR) (c) activity in the plasma of red striped snapper Lut*janus erythropterus* that is fed with AX- and/or OX supplemented diet. Letters: significant differences between treatments for the same parameter assayed ($p \leq 0.05$). (n = 6). Time elapsed: hour(s) after feeding the treatments.

concentration at 6 h as compared to non-OX-fed fish (C and AX). Significant interaction effect of AX and OX was only found on lactate at 6 h.

The plasma glucose, triglycerides, and lactate concentrations of fish fed with the four diets are shown in Fig. 2. AX-fish had lower glucose concentration than the OX-fish and C-fish at 6 h. Glucose concentration of AX-fish was comparable to the C-fish and AX + OX-fish at 12 h. Furthermore, Glucose concentration of OX-fish remained higher until 12 h. For triglycerides, significant difference was only found at 12 h. The AX-fish had lower triglyceride concentration than OX-fish. Similarly, lactate concentration of AX-fish was the lowest among treatments at 6 h. However, no changes were found in lactate concentrations of the rest of the sampling times. Glucose, triglycerides, and lactate concentrations returned to initial level at 24 h.

3. Liver AX Concentration

The liver AX concentration of AX- and AX + OX-fish was significantly higher than that of C and OX-fish (Table 3). Moreover, significant increase in diester, monoester, and free AX levels was also observed among fish fed with AX supplemented diet.

4. Correlation Analysis

Correlations among antioxidant capacity (SOD, GPx, and GR), metabolic response (glucose, triglycerides, and lactate)

Table 2. Average plasma metabolic response of red striped snapper Lutjanus erythropterus at 6, 12, 24, 48 and 72 h after being fed with AX- and/or OX-supplemented diet.

Time (h)	Parameters (mg/dl)	Astaxanthin[1] (AX)		Oxidized oil (OX)[2]		AX x OX Interaction[3] (Pr > F)	M.S.E.[4]
		No	Yes	No	Yes		
6	Glucose	133.93^{a} (5.79)	105.67^{b} (7.25)	110.87^{x} (6.70)	128.73^{x} (8.69)	0.50	1×10^{2}
	Triglycerides	157.37^{a} (6.46)	154.61^{a} (5.87)	152.67^{x} (4.20)	159.30^{x} (7.48)	0.85	1×10^{1}
	Lactate	9.19^{a} (0.70)	6.39^{b} (0.91)	6.19^{y} (0.19)	9.38^{x} (0.44)	0.05	9.24
12	Glucose	117.08^{a} (7.41)	90.50^{b} (4.37)	95.36^{y} (6.02)	112.22^{x} (8.24)	0.34	2×10^{2}
	Triglycerides	188.18^{a} (6.83)	163.44^{b} (6.94)	165.88^{y} (7.94)	185.74^{x} (6.88)	0.86	8.78
	Lactate	8.44^{a} (0.56)	8.57^{a} (0.45)	8.03^{x} (0.45)	8.98^{x} (0.50)	0.82	1×10^{-1}
24	Glucose	96.63^{a} (3.32)	88.49^{a} (4.02)	88.91^{x} (3.67)	96.20^{x} (3.83)	0.75	1×10^{1}
	Triglycerides	167.25^{a} (12.45)	166.18^{a} (11.06)	166.19^{x} (13.16)	167.24^{x} (10.19)	0.78	1×10^{2}
	Lactate	5.78^{a} (0.36)	5.28^{a} (0.35)	5.15^{x} (0.28)	5.92^{x} (0.39)	0.86	3×10^{-2}
48	Glucose	88.51^{a} (5.02)	79.52^{a} (4.64)	80.36^{x} (5.80)	87.66^{x} (3.89)	0.40	1×10^{1}
	Triglycerides	162.69^{a} (7.93)	165.50^{a} (5.86)	163.60^{x} (7.55)	164.58^{x} (6.38)	0.99	8×10^{-2}
	Lactate	5.79^{a} (0.38)	5.14^{a} (0.24)	5.27^{x} (0.24)	5.66^{x} (0.40)	0.20	1.43
72	Glucose	84.39^{a} (4.10)	83.15^{a} (3.34)	83.86^{x} (4.05)	83.68^{x} (3.42)	0.72	1×10^{1}
	Triglycerides	156.22^{a} (7.24)	153.98^{a} (7.40)	157.56^{x} (7.41)	152.64^{x} (7.12)	0.56	1×10^{2}
	Lactate	4.46^{a} (0.27)	5.13^{a} (0.18)	4.67^{x} (0.24)	4.92^{x} (0.28)	0.70	8×10^{-2}

Under the same main factor, means (S.E.) without a common superscript are significantly different ($p \leq 0.05$).

[1] Diets were supplemented with astaxanthin and with and without oxidized oil.

[2] Diets were supplemented with oxidized oil and with and without astaxanthin.

[3] Interaction between astaxanthin and oxidized oil.

[4] Mean standard error.

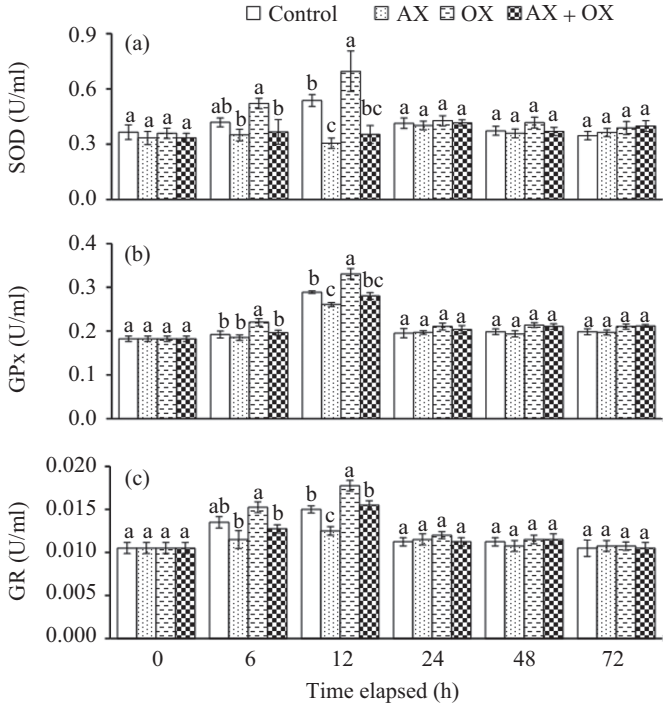

Fig. 2. Mean (S.E.) glucose (a), triglycerides (b) and lactate (c) levels in plasma of red striped snapper *Lutjanus erythropterus* fed diet with astaxanthin and/or oxidized oil. Letters: significant differences between treatments for the same parameter assayed ($p \leq 0.05$). (n = 6). Time elapsed: hour(s) after feeding the treatments.

Table 3. Astaxanthin concentration in liver of red striped snapper *Lutjanus erythropterus* at 72 h after feeding with a diet with AX and/or OX-supplemented diet.

Astaxanthin* (mg/kg)	Treatments			
	Control	AX	OX	AX + OX
DA	6.92 (0.19)^{b}	10.76 (0.10)^{a}	7.05 (0.24)^{b}	10.49 (0.54)^{a}
MA	24.86 (0.66)^{b}	38.65 (0.38)^{a}	25.14 (0.70)^{b}	37.17 (1.57)^{a}
FA	21.21 (0.44)^{b}	43.06 (5.35)^{a}	24.87 (1.54)^{b}	57.46 (11.73)^{a}
`TA	52.99 (1.29)^{b}	92.47 (4.86)^{a}	57.07 (2.48)^{b}	105.11 (9.63)^{a}

Means (S.E) without a common superscript between treatments are significantly different ($p \leq 0.05$). n = 6.

*Astaxanthin: DA-xdiester astaxanthin; MA- monoester astaxanthin; FA- free astaxanthin TA- total Astaxanthin.

and liver AX concentration (DA, MA, FA, and TA) were observed (Table 4). Among those correlations, DA and MA had negative correlations with SOD, GR, glucose and lactate at 6 h while glucose and triglycerides had positive correlations with FA at 12 h.

IV. DISCUSSION

OX in the diet results in higher activities of antioxidant enzymes such as SOD, GPx, and GR in fish. The present result corroborates the study of Chen et al. (2011) wherein large-

Table 4. Correlation matrix among antioxidant capacity, metabolic response and liver astaxanthin concentration of red striped snapper Lutjanus erythropterus fed diet with astaxanthin and/or oxidized oil.

Time[1]	6						12						72			
Parameters[2]	SOD	GPx	GR	GLU	TRI	LAC	SOD	GPx	GR	GLU	TRI	LAC	DA	MA	FA	TA
SOD		0.52*										0.67*	-0.57*	-0.57*		
GPx			0.54*													
GR				0.51*		0.61*							-0.54*	-0.56*		
GLU						0.56*							-0.59*	-0.61*	-0.63*	-0.68*
TRI																
LAC													-0.61*	-0.63*		
							SOD	0.78*	0.58*							
							GPx	0.70*	0.68*							
							GR	0.69*								
							GLU	0.67*							0.61*	
							TRI								0.68*	0.62*
							LAC									
													DA	0.99*	0.96*	0.84*
														MA	0.68*	0.85*
															FA	0.96*

The correlation between two parameters is shown by correlation coefficient (r) value; *significant ($p \leq 0.05$); Blank- not significant.

[1] hours after feeding the treatments.

[2] Parameters: SOD-Superoxide dismutase; GPx- Glutathione peroxidase; GR- Glutathione reductase; GLU- Glucose; TRI-Triglycerides; LAC-Lactate; DA- Diester astaxanthin; MA- Monoester astaxanthin FA- Free astaxanthin; TA- Total astaxanthin. Parameters on antioxidant capacity and metabolic response with or without underline were measured at 6 and 12 h after feeding treatment diet, respectively.

mouth bass *Micropterus salmoides* (Lacepède) fed with OX diets had higher liver antioxidant enzymatic activities than that of the fresh oil receiving group. Hence, the plasma antioxidant capacity in *L. erythropterus* could be weakened by feeding diets containing OX. However, AX + OX-fish had lower SOD, GPx, and GR activities than OX-fish. Thus, AX was able to modulate antioxidant capacity after feeding a diet with OX. This finding could be an indicator of improved fish welfare.

AX can enhance antioxidant capacity as reflected by decreasing SOD, GPx and GR under stress condition. In the present study, SOD, GPx, and GR activities of AX-fish were decreased by 56, 21, and 30% as compared to OX-fish 12 h, respectively. These results are in agreement with Pan et al. (2010a; 2010b) in characins *Hyphessobrycon callistus* (Steindachner). Moreover, SOD decreased with increasing dietary carotenoid concentrations (Wang et al., 2006). It is speculated that after feeding with AX, the increase in body AX concentration can result in better oxidation-reduction buffer capacity within the cells. The lower values recorded in each antioxidant capacity indicator could therefore be due to the development of the antioxidant defense system within a particular period of time. Consequently, the need to produce SOD to scavenge superoxide radicals was lessened. Lower GPx activity was observed when *H. callistus* were fed with pigmented diets, and the decreasing trend of GPx with increasing dietary carotenoid concentration showed that carotenoids could reduce peroxide in cells and concomitantly this enzyme activity (Wang et al., 2006). Additionally, GPx seems to be important to the metabolism of H_2O_2 at relatively lower steady-state of H_2O_2 concentrations (Polavarapu et al., 1998). Since AX contains a large conjugated double bond system with relatively unstable electron orbital, it scavenges oxygen radicals in the cells (Naguib, 2000), thereby minimizes cellular damage (Mourente et al., 2002).

Metabolic response such as glucose, triglycerides and lactate vary substantially among stressed and unstressed fish. Their levels are typically elevated when fish are under stress. In this study, glucose concentrations before feeding were relatively lower than after feeding, probably due to starvation prior to the onset of the experiment. Increased plasma glucose concentrations in response to stress stimuli would activate metabolic response of L. erythropterus to cover increased metabolite requirements associated with the classical stress caused by the OX. It is, however, noteworthy to mention that just like in the study of Alves-Martins et al. (2007), plasma glucose concentration of fish fed diet with OX increased significantly immediately after heat shock and decreased to pre-stress levels by 6 h. Furthermore, it has been reported that after 4 h exposure to hypoxia stress, there was an elevation (average ~ 75%) in plasma glucose concentration of wolfish Anarhichas minor (Olafsen) which indicated hyperglycemic response (Lays et al., 2009). Since L. erythropterus fed diet with AX had more stable glucose concentrations compared to the other treatments, it could indicate that AX could act as modulator of fish metabolic response.

Fish fed diet with antioxidant like AX and vitamin E stabilize triglycerides concentration under stress condition (Nakano et al., 1999; Trenzado et al., 2008). In this study, triglycerides concentration of AX-fish was decreased by 22% as compared to the OX-fish at 12 h. In other studies, it has been reported

that vitamin E deficiencies in fish has led to significant increase in triglycerides concentrations under crowding conditions (Trenzado et al., 2008). Furthermore, that *O. mykiss* fed diet with OX had higher triglycerides concentrations while triglycerides concentration of fish fed dietary red yeast *Phaffia rhodozyma,* which is rich in AX, was as low as that of the control (Nakano et al., 1999). The plasma triglycerides concentration-lowering effect of AX is attributed to its antioxidative action which normalizes the dysfunction of the lipid metabolism in the liver (Nakano et al., 1999).

The supplementation of carotenoids, such as AX, into the feed raises the liver AX concentration of fish. AX deposited mainly in the liver plays an important role in the absorption of dietary carotenoids (Ytrestøyl and Bjerkeng, 2007). This organ has been considered as the site of carotenoid storage and the pathway of pigment transfer into the blood. Thus, strong antioxidant defense systems are evolved in the liver to cope with ROS (Xi and Chen, 2000).

Significant correlations were observed between antioxidant capacity and liver AX concentration. Significant correlations among SOD, GPx and GR could indicate that the protective defense of SOD against superoxides was related to the removal of H_2O_2 by GPx (Pan et al., 2010a) and removal of toxic metabolites by GR. GR activity is also directly implicated with GPX activity since it provides regenerated GSH to GPX. An increase in the amount of free radicals and H_2O_2 would logically predict a compensatory increase in enzyme activity to overcome oxidative stress (Polavarapu et al., 1998). A synergistic relationship was also observed between SOD and GPx in *H. callistus* under hypoxia stress (Lushchak et al., 2001; Pan et al., 2010a). Furthermore, SOD and GPx are found to be negatively correlated to body AX concentration (Wang et al., 2006) which was also observed in this study where SOD and GR had negative correlations with liver AX concentration. The increase in body carotenoid concentration could enhance oxidation-reduction buffer capacity within the cells (Latscha, 1990; Oshima et al., 1993) that could inhibit singlet oxygen reaction, which is induced by the stress (Di Mascio et.al., 1991). Hence, the need to produce SOD to scavenge superoxide radicals is lessened.

Significant correlations between metabolic response and liver AX concentration can be influenced by different factors. Among metabolic responses, the positive correlations between glucose and lactate and their negative correlations with liver AX concentration at 6 h could imply that more stable metabolic response (lower glucose and lactate concentration) could be regulated by a higher liver AX concentration of *L. erythropterus*. In other study, glucose, triglycerides and lactate concentrations decreased with the increase of dietary AX (Li et al., 2014). Thus, AX besides its antioxidative role, it also acts as a modulator of classical stress response. In contrast, glucose and triglycerides concentrations had positive correlations with liver AX concentration at 12 h. Variation on the correlation of antioxidant capacity and metabolic response to liver AX concentration at 6 h and 12 h might have been due to the single data set used in liver AX concentration which is obtained at 72 h. It is interesting to note that the relationship of antioxidant capacity and metabolic response to liver AX concentration at 6 h was more sensitive than 12 h since it was recorded that former has more significant correlations (9) than the latter (3). Moreover, glucose concentration at 6 h was negatively correlated to DA, MA, FA and TA while glucose concentration at 12 h was only positively correlated to FA which was less than 50% of the TA. A minimal increase of glucose and triglycerides concentrations within the normal range together with the increasing liver AX concentration may still be due to the enhancement of fish health status (Abdel-Tawwab et al., 2007). The variation of the correlations between 6 and 12 h is still hard to explain since no other report is available on the correlation of metabolic responses on AX concentration and health status of fish.

V. CONCLUSION

Overall, supplementation of AX in the diet significantly stabilizes both antioxidant capacity and metabolic responses of *L. erythropterus* under a prooxidant circumstance associated with oxidized fish oil intake. This study opened an avenue for further exploration on the optimal use of dietary AX as antioxidant to improve fish welfare. The different responses of fish observed by feeding OX and antioxidant such as AX are still unclear and need to be further investigated. Considering the result from this study, *L. erythropterus* can elicit significant differences on antioxidant capacity and metabolic responses when fed with an AX and/or OX-supplemented diet. Extended feeding at lower AX dosage is worth studying as a stress relief measure and means to maintain homeostasis.

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